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The Sequences of Five Neuropeptides Isolated from *Limulus* using Antisera to FMRFamide

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Abstract. Five neuropeptides were isolated from CNS extracts of the horseshoe crab *Limulus polyphemus* by high pressure liquid chromatography (HPLC). The peptides were identified by radioimmunoassays (RIAs) based on two antisera raised to FMRFamide-related peptides (FaRPs). The purified peptides were analyzed by automated sequencing and mass spectrometry, and the following sequences were obtained: DEGHKMLYFamide, GHSLLHFamide, PDHHMMYFamide, DHGNMLYFamide, and GGRSPSLRLRFamide. The first four peptides are members of a novel family with virtually no relationship to FMRFamide. GGRSPSLRLRFamide, on the basis of structural similarity, becomes the second member of a class of FaRPs known previously only from a peptide isolated from mosquito heads. At least one member of the novel family (GHSLLHFamide) inhibits the isolated heart of *Limulus*.

Introduction

The large set of peptides related—by one or more traits, and to a greater or lesser extent in each—to the molluscan peptide FMRFamide (Phe-Met-Arg-Phe-NH_2) is distributed throughout the animal kingdom, and FMRFamide-like immunoreactivity, as revealed by immunocytochemistry, is seemingly ubiquitous as well (for review see Price and Greenberg, 1989; Greenberg and Price, 1992).

In the horseshoe crab *Limulus polyphemus,* prominent FMRFamide-like immunoreactivity occurs in the brain, cardiac ganglion, and ventral nerve cord (Watson *et al.*, 1984; Watson and Groome, 1989; Groome, 1991). In addition, efferent axons that project to the lateral eyes of *Limulus* also contain FMRFamide-like immunoreactivity (Lewandowski *et al.*, 1989), though these fibers seem to be part of a general epidermal innervation rather than being restricted to the ommatidia. In any event, none of the peptides responsible for this immunoreactivity in *Limulus* have been sequenced.

Biological activity by FMRFamide-related peptides (FaRPs) has also been demonstrated in the horseshoe crab. In particular, two peptides isolated from the lobster (*Homarus americanus*)—F1 (Thr-Asn-Arg-Asn-Phe-Leu-Arg-Phe-NH_2; TNRNFLRFamide) and F2 (Ser-Asp-Arg-Asn-Phe-Leu-Arg-Phe-NH_2, SDRNFLRFamide; Trimmer *et al.*, 1987)—increased both the amplitude and rate of beat of isolated *Limulus* hearts. These effects were similar to those of a partially purified *Limulus* brain extract (Groome, 1991).

Because analogs of F1 and F2 occur in other crustaceans and in insects (Greenberg and Price, 1992), one might suppose that the FMRFamide-like immunoreactivity and cardioactivity in *Limulus* is due to similar peptides. But arthropods contain more than one class of FMRFamide-related peptides. Indeed, in insects, which have been much more extensively and intensively studied, each species appears to have at least four distinct classes of FaRPs (Table I). Moreover, members of the different classes have different biological effects. For example, leucosulfakinin excites the cockroach hindgut while leucomyosuppressin inhibits it (Nachman *et al.*, 1986; Holman *et al.*, 1986); similarly, leucomyosuppressin and its locust homolog SchistoFLRFamide inhibit the semi-isolated heart of *Schistocerca,* whereas F1 and FMRFamide itself are car-

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Table 1

*Representatives of four classes of FMRFamide-related peptides present in dipteran insects**

Class	Sequence	Reference
FaGRPs[a]	Asp-Pro-Lys-Gln-Asp-Phe-Met-Arg-Phe-NH_2	(Schneider and Taghert, 1988)
Myosuppressins[b]	Thr-Asp-Val-Asp-His-Val-Phe-Leu-Arg-Phe-NH_2	(Nichols, 1992)
Sulfakinins[c]	pGlu-Ser-Asp-Asp-Tyr-Gly-His-Met-Arg-Phe-NH_2	(Nichols *et al.*, 1988)
Head peptides[d]	pGlu-Arg-Pro-Pro-Ser-Leu-Lys-Thr-Arg-Phe-NH_2	(Matsumoto *et al.*, 1989)

* The first three peptides in the list were isolated from *Drosophila melanogaster*; the fourth is from *Aedes aegypti*.

[a] FMRFamide-gene-related peptides; multiple, variable copies processed from a precursor.

[b] Similar C-terminal tetrapeptide, but not encoded on any known FMRFamide gene; peptide shown is *Drosophila* myosuppressin; type of the class is leucomyosuppressin from *Leucophaea maderae*.

[c] Only C-terminal tripeptide is analogous to FaGRPs; peptide shown is drosulfakinin; type of the class is leucosulfakinin.

[d] Only C-terminal dipeptide is analogous to FaGRPs.

dioexcitatory (Cuthbert and Evans, 1989; Robb *et al.*, 1989).

We therefore isolated the FMRFamide-like immunoreactivity in *Limulus* brains and circumesophageal rings and report here the sequences of five neuropeptides that were identified. One is homologous to an insect head peptide and is only the second member of what may be an arthropodan class of FaRPs. The other four are apparently homologs in a novel peptide family of uncertain phyletic relations. One peptide from this novel group was synthesized and tested on the isolated *Limulus* heart; it is not only a potent cardioinhibitor, but also occurs in heart extracts. We speculate, therefore, that this peptide functions as a cardioregulatory neurotransmitter.

Materials and Methods

Animals

Adult *Limulus polyphemus*, in intermolt and measuring 15–25 cm across the carapace, were collected in the Indian River near Cape Canaveral, Florida. The animals were maintained in running natural seawater (15–18°C) and fed once a week.

Peptide extraction and purification

Brains (including both the protocerebrum and the circumesophageal ring) were collected from horseshoe crabs over several months as the animals were used for visual system studies. Each excised piece of tissue was weighed and then added to a flask of acetone (40 ml) that was kept at −20°C between successive additions. When 9–10 g had been accumulated, the extract was further processed as outlined below. Heart (12.9 g) and lateral eye (3.5 g) were treated similarly.

The acetone extracts were decanted from the pieces of tissue, then clarified by centrifugation, and reduced in volume on a rotary evaporator. The latter step removed most of the acetone and left an aqueous phase consisting of water derived from the tissue. After the addition of 50 ml aqueous 0.1% trifluoroacetic acid (TFA), the extract was again clarified by centrifugation and by filtration through a nylon filter (0.45 μm pore size). The clarified liquid was pumped onto an HPLC column (Brownlee C8 RP-300, 4.6 mm × 220 mm) at 2 ml/min, and this flow rate was maintained throughout the run. After the column had been loaded, it was washed with 0.1% aqueous TFA until the absorbance returned close to the baseline. The solvent composition was then stepped to 16% acetonitrile (ACN) in aqueous 0.1% TFA, and a linear gradient (0.8%/min increase in ACN concentration) was begun when the solvent front reached the detector as indicated by a rapid increase in absorbance. Fractions were collected by time (0.5 min), and an aliquot (2 μl) of each fraction was taken for radioimmunoassay (RIA).

Further purifications of immunoreactive fractions were performed on a smaller diameter column containing the same packing as that used for the initial run (Brownlee C8 RP-300; 2.1 × 220 mm), but with two additional solvent systems: 80% ACN containing 0.05% heptafluorobutyric acid (HFBA), or phosphate buffer (5 m*M*) containing 60% ACN, pH 7.0. The gradients were 0–30, 10–40, or 20–50% of the respective organic solvent, over 30 min, at a flow rate of 0.5 ml/min. The pooled immunoreactive fractions from one run were diluted with an aliquot of aqueous solvent and pumped onto the column at 0.5 ml/min for the next run. Once a peak was substantially pure, we attempted to oxidize the contained peptide and thereby shift its elution time; this maneuver was meant to achieve a further purification, and to help in the characterization. We oxidized the peptide by addition of hydrogen peroxide (50 μl/ml of a 30% solution) directly to the immunoreactive fraction in a TFA- or HFBA-containing solvent; this converts methionine almost

exclusively to methionine sulfoxide. The reaction was terminated after 15 min by the addition of 1–2 ml aqueous solvent, and the sample was loaded onto the HPLC column.

Radioimmunoassay (RIA)

RIAs were carried out as previously described (*e.g.*, Price, 1982; Price *et al.*, 1987, 1990). Two antisera that had been raised in rabbits to thyroglobulin-peptide conjugates were used in these assays: antiserum S253 raised to YGGFMRFamide, and Q2 raised to pGluDPFLRFamide and AspDPFLRFamide. Iodinated pQYPFLRFamide was the trace for both assays.

Mass spectrometry and sequencing

The molecular ions and, in some cases, fragment ions of purified immunoreactive fractions were analyzed by fast atom bombardment mass spectrometry (FABms), as described previously (Bulloch *et al.*, 1988). Fractions were also subjected to automated Edman sequencing (Applied Biosystems Inc. sequencer, Model 470A) with automatic, online HPLC analyses of the phenylthiohydantoin (PTH) amino acids released at each cycle (performed by B. Parten, University of Florida Interdisciplinary Center for Biotechnology Research, Protein Chemistry Core Facility, Gainesville. See Díaz-Miranda *et al.*, 1992, for details).

Synthetic peptides

Peptide resins (GHSLLHFamide and GGRSPSLRLRFamide) were synthesized by the Protein Chemistry Laboratory of the University of Florida's Interdisciplinary Center for Biotechnology Research on an Applied Biosystems 430A peptide synthesizer, starting with MBHA resin and utilizing the protecting groups recommended by Applied Biosystems for use with the trifluoromethanesulfonic acid (TFMSA) deprotection and cleavage system. The peptides were deprotected and cleaved from the resin at the Whitney Laboratory (with TFMSA), purified by HPLC, and quantified by amino acid analysis (Hitachi 835 analyzer).

Bioassay

The heart with its associated cardiac ganglion was removed from the animal and suspended lengthwise in a glass chamber (25 ml) containing natural seawater that was aerated continuously. One end of the heart was fixed near the bottom of the chamber, and the opposite end was hooked to a force-displacement transducer (Grass Model FT .03C). The amplitude and frequency of heartbeat was displayed on an ink-writing oscillograph (Grass Model 7 Polygraph).

Synthetic peptides (GGRSPSLRLRFamide and GHSLLHFamide) were dissolved in an aliquot of seawater (200 μl) which was added directly to the chamber; concentrations are expressed as moles per liter in the bath. The amplitude of beat was measured at the point of maximal response, about 2–3 min after the dose of peptide was added. This measurement, expressed as a percentage of the beat amplitude before treatment, was taken as the response.

Results

Identification of peptides from the CNS

Two CNS extracts were chromatographed by HPLC, and the fractions were assayed with both the Q2 and S253 antisera. The fractions constituting corresponding immunoreactive peaks from each chromatographic run were combined and further purified. In the end, six peaks that were immunoreactive with the Q2 antiserum were isolated.

One of these peaks showed slight immunoreactivity with the S253 antiserum (in addition to that with Q2), did not undergo a shift in elution time after oxidation, and showed very little UV absorbance at 280 nm. We had sufficient material to obtain both a molecular ion and sequence data from this peak. From the protonated molecular ion, which had an m/z ratio of 1244.6, and the sequence Gly-Gly-Arg-Ser-Pro-Ser-Leu-Arg-Leu-Arg-Phe, we deduced the full structure GGRSPSLRLRFamide. This sequence is very similar to that of Aea-HP, the mosquito head peptide (Matsumoto *et al.*, 1989; see Table I), so we will refer to it henceforth as the *Limulus* head peptide (Lip-HP)*.

A second immunoreactive peak (LP1)—after oxidation—eluted about 3 min earlier and showed significant UV absorbance at 280 nm. This peak yielded a partial sequence XEGHKMLYF (X-Glu-Gly-His-Lys-Met-Leu-Tyr-Phe), where X could have been Ala, Asp, or Ser. Although this sequence did contain the methionine and aromatic amino acid (tyrosine, Y) expected from the properties of the immunoreactive peptide in peak LP1, it remained ambiguous, not only because of the free amino acid contamination, but also because no prominent molecular ion was found.

Analysis of the third peak (LP2) revealed a prominent monoisotopic, protonated ion at an m/z ratio of 809.4, and subsequent sequencing of the peak yielded the peptide

* Of course *Limulus* lacks a head, but we retain the name "head peptide" for the sake of continuity and a modest reduction in confusion. The species designation, as with some insect peptides, is derived from the first two letters of the genus and the first letter of the species; hence "Lip" from *Li*mulus *p*olyphemus.

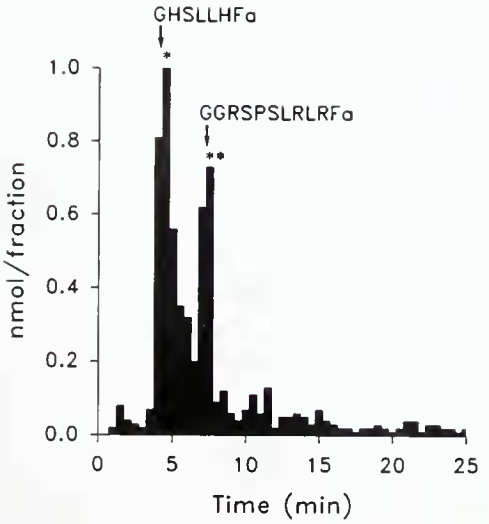

Figure 1. RIA analysis of the initial HPLC fractionation of a *Limulus* CNS extract (second experiment). Half-minute fractions were collected, and 2-μl aliquots were taken from each fraction for the RIA using the Q2 antiserum. The immunoreactive peaks containing peptides LP1-LP4 are indicated: * (LP1, LP2, LP3); ** (LP4). In a previous purification, the *Limulus* head peptide (Lip-HP) was isolated from the second peak (**). The elution times of LP2 (GHSLLHFamide) and Lip-HP (GGRSPSLRLRFamide) are indicated with arrows. The sequences and molecular ions of all of the peptides are listed in Table II.

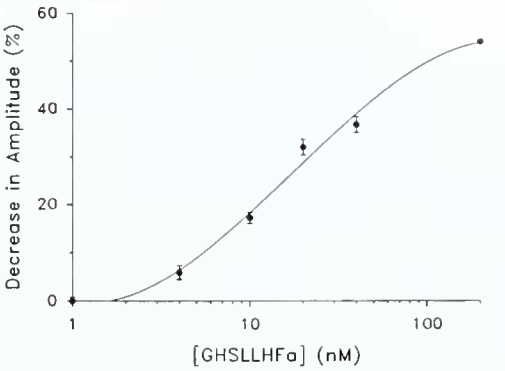

Figure 2. Dose-response relationship of the effect of GHSLLHFamide (LP2) on the isolated *Limulus* heart. LP2 decreases the amplitude of the beat with a threshold of 4 n*M*. (Nine or ten determinations on five different hearts; SEM < 2%). The measurement of the response, the percentage of amplitude relative to that of the control beat, is described in Materials and Methods.

sequence GHSLLHF. This sequence is in agreement with the calculated molecular weight of 808 for GHSLLHF, assuming an amidated carboxyl terminus (Gly-His-Ser-Leu-Leu-His-Phe-NH_2).

The remaining three peaks contained insufficient material for either microsequencing or FABms. We therefore prepared a new extract to resolve the ambiguities left by the first experiment.

The first HPLC fractionation of this new CNS extract, after RIA analysis with the Q2 antiserum, showed immunoreactive peaks (Fig. 1) similar to those in the RIA profile obtained from the earlier CNS extracts. When the S253 antiserum was used, some peaks were more clearly defined than in the previous experiment. Two peptides (LP3 and LP4) that reacted only with the Q2, and that had not been identified earlier, were isolated and identified. In addition, the identification of LP1 was completed with the new extract. The details of these findings are set out below.

After oxidation, peak LP3 eluted almost 8 min earlier; shifts of this magnitude are indicative of more than one methionyl residue. A prominent monoisotopic protonated ion was found at an m/z ratio of 1109.2, and automated Edman degradation of 125 pmol of peptide yielded the sequence PDHHMMYF (Pro-Asp-His-His-Met-Met-Tyr-Phe). This sequence is consistent with the calculated molecular weight of 1108 for LP3, assuming an amidated carboxyl terminus and both methionyl residues as the sulfoxides.

The elution time of LP4 also shifted after oxidation, though not as much as that of LP3. FABms analysis of the immunoreactive peak revealed a prominent protonated molecular ion at an m/z ratio of 1011.2. Further analysis of the fragment ions yielded the sequence DHGNMLYFamide (Asp-His-Gly-Asn-Met-Leu-Tyr-Phe-NH_2).

Finally, the molecular ion for LP1 was obtained (1155.8), and fragment ions indicative of an N-terminal aspartic acid residue were also found. So the sequence of this peptide was deduced to be: DEGHKMLYFamide.

Peptide synthesis

The synthetic peptides GGRSPSLRLRFamide and GHSLLHFamide were purified by HPLC. Amino acid

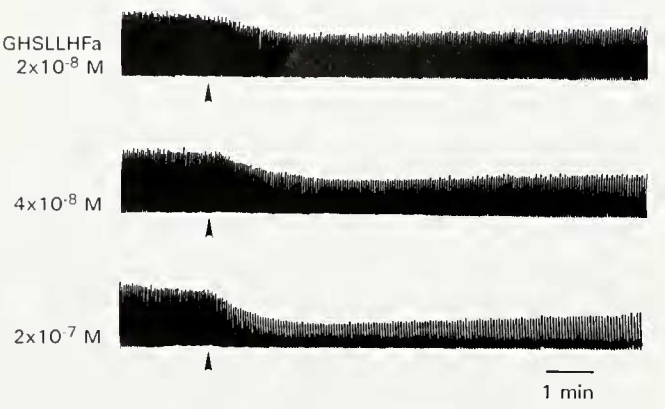

Figure 3. LP2-induced decreases in strength and rate of contractions of the isolated *Limulus* heart. Whereas the effect on the heart rate is long-lasting, the amplitude starts to increase again 4–5 min after peptide application.

Table II

Peptides isolated and sequenced from Limulus

Name	Sequence	Monoisotopic MH+**	Average MH+	Observed MH+
Lip-HP*	GGRSPSLRLRFamide	1244.73	1245.48	1244.6
LP1	DEGHKMLYFamide	1154.53	1155.32	1155.8 (Avg)
LP2	GHSLLHFamide	809.44	809.95	809.4
LP3	PDHHMMYFamide	1108.45	1109.27	1109.2 (Avg)
LP4	DHGNMLYFamide	1011.44	1012.13	1011.2

* Lip-HP: *Limulus* head peptide (see footnote *; p. 324).
** MH+; protonated molecular ion, nominal mass.

analysis of an aliquot gave the following composition of GGRSPSLRLRFamide: Gly 2.2, Ser 1.9, Leu 2.2, Phe 0.8, Pro 1.0, Arg 3.0. Amino acid analysis of an aliquot of GHSLLHFamide yielded the composition: Gly 1.1, Ser 1.0, Leu 2.0, Phe 0.86, His 1.6. These synthetic peptides co-eluted with their natural congeners when they were rechromatographed on the 2.1 × 220 mm Brownlee column with the ACN/TFA solvent system used with a gradient of 20–50%.

Peptides from the heart

The heart contains less peptide per unit weight than the CNS, but we were still able to purify enough of one Q2-reactive peak to carry out an FABms analysis. This peak showed a prominent protonated molecular ion at an m/z ratio of 809 (the same as LP2), and fragment ions characteristic of the sequence GHSLLHFamide were observed.

Lateral eye extract

HPLC purification of a lateral eye extract, and subsequent analysis by RIA with the Q2 and S253 antisera, revealed no significant immunoreactivity.

Bioactivity of synthetic GGRSPSLRLRFamide and GHSLLHFamide

Concentrations of synthetic GGRSPSLRLRFamide between 2×10^{-8} and 10^{-6} mol/l had no effect on the rate or amplitude of isolated *Limulus* hearts. A slight decrease in amplitude was finally observed at 5×10^{-6} mol/l, but no dose-response curve could be established for this peptide.

Synthetic GHSLLHFamide, on the other hand, produced a dose-dependent decrease in beat amplitude; the threshold was 4×10^{-9} mol/l. Figure 2 shows the dose response relationship for this response; each point represents the average decrease in amplitude from 9–10 experiments with five different hearts. Higher concentrations of GHSLLHFamide (from 2×10^{-8} mol/l) also decreased heart rate (Fig. 3).

5-HT (5-hydroxytryptamine, serotonin) also inhibits the *Limulus* heart, and its effects are blocked by 2-bromo, d-lysergic acid diethylamide (Burgen and Kuffler, 1957; Pax and Sanborn, 1967). We asked whether another lysergic acid analog, methysergide [a 5-HT antagonist on molluscan (Wright *et al.*, 1962) and crustacean (Kerkut and Price, 1964) hearts] might block the effect of

Table III

Comparison of Limulus *head peptide (Lip-HP) with peptides from other species*

Peptide*	Species	Sequence	Reference
Lip-HP	*Limulus polyphemus*	Gly-Gly-Arg-Ser-Pro-Ser-Leu-Arg-Leu-Arg-Phe-amide	This paper
Aea-HP	*Aedes aegypti*	pGlu-Arg-Pro-Pro-Ser-Leu-Lys-Thr-Arg-Phe-amide	(Matsumoto *et al.*, 1989)
NPF	*Helix aspersa*	-Tyr-Ala-Ile-Met-Gly-Arg-Thr-Arg-Phe-amide	(Halton *et al.*, 1992)
PP (C-terminal)	*Alligator mississippiensis*	-Tyr-Leu-Asn-Val-Val-Thr-Arg-Pro-Arg-Phe-amide	(Lance *et al.*, 1984)
—	*Helicoverpa zea*	Gln-Ala-Ala-Arg-Pro-Arg-Phe-amide	(Crim, pers. comm.)
NPFF	*Bos taurus*	Pro-Phe-Trp-Ser-Leu-Ala-Ala-Pro-Gln-Arg-Phe-amide	(Yang *et al.*, 1985)
Chicken brain peptide	*Gallus gallus*	Leu-Pro-Leu-Arg-Phe-amide	(Dockray *et al.*, 1983)

* Abbreviations: Aea-HP, *Aedes aegypti* head peptide; NFP, neuropeptide F; PP, pancreatic polypeptide (NFP and PP sequences are not complete; only the C-terminal is shown.); NPFF, neuropeptide Phe-Phe; (—), no common name given.

Table IV

Sequence homologies of peptides from Limulus, *a chiton, and a sea cucumber*

Species		Sequence
Limulus polyphemus	(LP1)	Asp-Glu-Gly-His-Lys-Met-Leu-Tyr-Phe-amide
	(LP2)	Gly-His-Ser-Leu-Leu-His-Phe-amide
	(LP3)	Pro-Asp-His-His-Met-Met-Tyr-Phe-amide
	(LP4)	Asp-His-Gly-Asn-Met-Leu-Tyr-Phe-amide
*Acanthopleura granulata**		Gly-Gly-Thr-Leu-Leu-Arg-Phe-amide
		Gly-Ser-Leu-Leu-Arg-Phe-amide
*Holothuria glaberrima***		Ser-Gly-Tyr-Ser-Val-Leu-Tyr-Phe-amide
		Gly-Phe-Ser-Lys-Leu-Tyr-Phe-amide

* A chiton; sequence from Greenberg and Price (1992).

** A sea cucumber; sequence from Díaz-Miranda *et al.* (1991).

GHSLLHFamide on *Limulus* heart. Methysergide alone increased the heart rate, but had no effect on the amplitude of beat. After application of methysergide (4×10^{-5} M) for 3 min, the inotropic effect of 5-HT (up to 2×10^{-7} mol/l) was totally blocked. In contrast, GHSLLHFamide, under the same conditions, still produced a dose-dependent decrease in amplitude. 5-HT and the peptide were each tested 10–11 times on 7 different hearts.

Discussion

We have isolated five neuropeptides from CNS extracts of the horseshoe crab *Limulus polyphemus* (Table II). One of these, GGRSPSLRLRFamide (Lip-HP), has 60–67% sequence similarity to another peptide, pQRPPSLKTRFamide (Aea-HP in Table III), which was discovered in the head of the mosquito *Aedes aegypti* (Matsumoto *et al.*, 1989). The identical residues are, furthermore, distributed along the length of the two sequences, and both peptides occur in arthropods. We therefore suppose that Lip-HP and Aea-HP are homologous.

The *Limulus* and mosquito head peptides also share some sequence similarity to the extended family of pancreatic polypeptide-like peptides (NPF and PP in Table III; see Halton *et al.*, 1992), as well as to a mélange of others (Table III). The disparity in the lengths of these peptides, the diversity of the organisms that contain them, and the restriction of the sequence similarities to the C-terminal pentapeptide, all suggest, however, that such similarities may be due to convergence.

The function of neither the horseshoe crab nor the mosquito head peptide is known. But since GGRSPSLRLRFamide had only a very weak cardioinhibitory effect in *Limulus*, with a threshold 5 μM, it is probably not a cardioregulatory agent.

An amidated carboxyl terminal phenylalanine is the only absolutely conserved feature shared by the *Limulus* peptides: DEGHKMLYFamide (LP1), GHSLLHFamide (LP2), PDHHMMYFamide (LP3), and DHGNMLYFamide (LP4). Nevertheless, histidine and tyrosine are commonly exchanged for one another in related proteins (their codons differ at only one position), and methionine-leucine exchanges are also quite frequent, as for example in FMRFamide and FLRFamide. We therefore tentatively propose that these four peptides represent a previously unknown, novel family in arthropods. Because the peptides are of roughly equal abundance, analogy with other peptide families (Greenberg and Price, 1992) suggests that they may even be products of a single precursor.

As one might expect of a "novel" family, the affinities of LP1-4 to other peptides are not obvious. Still, their amino acid sequences are somewhat similar to those of two peptides (GSLLRFamide and GGTLLRFamide) isolated from the granular spiny chiton *Acanthopleura granulata* (Greenberg and Price, 1992), and to another pair (GFSKLYFa and SGYSVLYFa) recently isolated from a sea cucumber, *Holothuria glaberrima* (Díaz-Miranda *et al.*, 1991) (Table IV). But the significance of these similarities is obscure.

One peptide of the proposed new family, GHSLLHFamide, was found in *Limulus* heart extracts. Synthetic GHSLLHFamide decreased the amplitude and rate of heartbeat, and the inotropic effect had a low threshold. The only other known cardioinhibitory transmitter in *Limulus* is 5-HT (Pax and Sanborn, 1967). The action and time course of 5-HT and GHSLLHFamide are quite similar, but whereas the effect of 5-HT can be completely blocked by methysergide, that of GHSLLHFamide was unaffected. We therefore conclude that the peptide does not act by releasing 5-HT from nerve terminals in the heart.

The activities of the other peptides, LP1, LP3, and LP4, are now being assayed on the *Limulus* heart. These experiments may reveal whether the sequence similarities between these peptides and GHSLLHFamide are more important functionally than their differences.

The two antisera used for RIA have quite different specificities for recognizing FaRPs; nevertheless, we are aware that many known FaRPs would not be detected by these antisera. A particular limitation is that the specificity of neither antiserum is determined primarily by the C-terminal RFamide, which is often thought to be the minimal determinant for a peptide to be considered FMRFamide-like. But the antisera do recognize well those peptides that contain the full C-terminal sequence: F(M or L)RFamide, especially when they are extended at the N-terminal. So it is surprising that we could isolate *no* such peptides, especially considering that their presence

has been established in other arthropods. However, because the various FaRPs present in any one species are not expressed at the same level, we may have missed some of the less abundant, but more FMRFamide-related peptides that may be present in *Limulus*. Indeed, evidence from bioassay and immunocytochemistry indicates that peptides more closely related to FMRFamide are present in *Limulus* (Watson *et al.*, 1984; Watson and Groome, 1989; Groome, 1991).

Notwithstanding that FaRPs have been detected immunocytochemically in the lateral eye of the horseshoe crab (Lewandowski *et al.*, 1989), we could detect no immunoreactive fractions in an extract of 50 lateral eyes. We therefore conclude that these peptides are present in the eye in such small amounts, that we are not able to detect them with the S253 and Q2 antisera.

In summary, we have isolated several members of a novel family of peptides, and one of them may be involved in cardioregulation. We have also isolated a close analog of an insect peptide, thereby establishing a pan-arthropodan class of FaRPs. The relationship between these two groups of peptides and those of other phyla are uncertain.

To date, only one other peptide from *Limulus* has been sequenced: proctolin (Groome *et al.*, 1990), which is identical in structure to the peptide originally found in the cockroach (H-Arg-Tyr-Leu-Pro-Thr-OH) by Starrat and Brown (1975). The effect of proctolin on the isolated *Limulus* heart is to increase the amplitude of contraction (Groome *et al.*, 1990).

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